Induction of 6a-Hydroxymaackiain 3-*O*-Methyltransferase and Phenylalanine Ammonia-Lyase mRNA Translational Activities during the Biosynthesis of Pisatin

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The isoflavonoid phytoalexin pisatin is synthesized by pea (*Pisum sativum* L.) in response to microbial infection and certain other forms of stress. The terminal step in the biosynthesis of pisatin is catalyzation by the (+)-6a-hydroxymaackiain 3-*O*-methyltransferase (HMKMT). This enzyme, identified as a protein of M_r 43,000 by photoaffinity labeling (Preisig *et al.* (1989) *Plant Physiol.* 91, 559–566), was purified 280-fold from $CuCl_2$-stressed pea seedlings and subjected to preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibodies were raised in rabbit against this protein cut from the polyacrylamide gels. The antiserum against the purified enzyme inhibited HMKMT enzyme activity and showed high specificity for the M_r 43,000 protein on Western blots and in immunoprecipitations. This enzyme, present almost exclusively in the 95,000*g* supernatant after differential centrifugation, was induced in pea from a low constitutive level by treatment with $CuCl_2$, suggesting that the HMKMT is newly synthesized in response to stress. HMKMT mRNA translational activity increased in peas with time after treatment with $CuCl_2$. Peak translational activity occurred about 12 h after treatment, preceding peak enzyme activity by a few hours. Phenylalanine ammonia-lyase (PAL) mRNA abundance increased coordinately with that of HMKMT. The increase in PAL mRNA translational activity in response to stress is known to reflect transcriptional activation of PAL genes. Thus, the induction by stress of enzyme activity both at an early step and at the terminal step in the phenylpropanoid/isoflavonoid biosynthetic pathway appears to be at the transcriptional level.

Many plants respond to microbial infection by synthesizing and accumulating low molecular weight toxic compounds, called phytoalexins, at the site of challenge. Pisatin, the major phytoalexin of garden pea (*Pisum sativum*), appears to have a role as a determinant of disease resistance in pea: only isolates of the root rot fungus *Nectria haematococca* that can detoxify pisatin show significant virulence on pea (1). The presence of pisatin is therefore suggested to contribute to the resistance of pea to nondetoxifying isolates of *N. haematococca* and possibly to other nondetoxifying fungi as well.

According to the biosynthetic pathway deduced from labeling studies *in vivo* (2), the terminal step for pisatin synthesis is the 3-*O*-methylation of 6a-hydroxymaackiain (HMK).[3] Recently, we reported the detection in pea of this methyltransferase. It was inducible by fungal infection or by treatment with $CuCl_2$ (3). The methyltransferase was identified as a protein of M_r 43,000 by photoaffinity labeling (4).

Biosynthesis of isoflavonoid phytoalexins such as pisatin has been the subject of much recent interest as a model system for studying the regulation of gene expression in plants (5). Although several enzymes specific for the isoflavonoid branch of the phenylpropanoid pathway have now been detected (3, 4, 6–11), studies at the nucleic acid level have been reported only for earlier enzymes of the general phenylpropanoid and flavonoid pathways. These earlier enzymes, in addition to functioning as the

first steps in the synthesis of isoflavonoid phytoalexins, provide the precursors of lignin and flavonoid pigments. Purification of isoflavonoid specific enzymes and cloning of their genes will allow comparison of their regulation with those enzymes that act earlier in the phenylpropanoid pathway.

In this paper, we present the characterization of polyclonal antiserum against the HMKMT and use it to confirm the exclusive presence of this enzyme in the soluble fraction after centrifugation of pea tissue extracts at 95,000*g*. The antisera were used in *in vitro* translation/immunoprecipitation studies to demonstrate the induction of HMKMT mRNA translational activity following treatment of pea seedlings with $CuCl_2$. Phenylalanine ammonia-lyase (EC 4.3.1.5), which catalyzes the first committed step of the phenylpropanoid pathway, was included in the study of mRNA translational activities as a reference for the time of induction of the HMKMT.

MATERIALS AND METHODS

Chemicals. AdoMet, rabbit IgG, and Protein A–Sepharose were purchased from Sigma. [*Me*-^{14}C]AdoMet, *trans*-^{35}S label, and nuclease-treated rabbit reticulocyte lysate were purchased from ICN Biomedicals, Inc.,[4] or Amersham. Phenol (molecular biology grade) was from Boehringer-Mannheim Biochemicals. SDS–PAGE blue-stained molecular weight markers, the alkaline phosphatase substrates 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium, and goat anti-rabbit IgG alkaline phosphatase conjugate were purchased from BioRad Laboratories. Published procedures were used to obtain (+)-HMK (4). Antiserum raised in rabbit against PAL from *P. sativum* (12) was the generous gift of Dr. L. Hadwiger.

Enzyme preparation and assay. Pea seeds (cv Alaska) were surface sterilized and germinated in the dark as described previously (4). After 5 to 7 days, seedlings were immersed in 5 mM $CuCl_2$ for 1 h, drained, and incubated for additional time as indicated. All steps of enzyme purification were carried out at 4°C. Protein determinations during enzyme purification were done by the method of Bradford (13) and during the time course and cellular fractionation experiments by the method of Markwell *et al.* (14). Time course and cellular fractionation experiments were conducted three times each.

For preparative scale experiments, roots and shoots (minus cotyledons) were frozen at −80°C 24 h after treatment with $CuCl_2$. The tissue was ground at 4°C in a blender in buffer (5 ml g^{-1} fresh weight) containing 14 mM 2-mercaptoethanol, 10 mM diethyldithiocarbamate, 5 mM EDTA, and 0.2 M potassium phosphate, pH 7.5, with 6% (w/v) polyvinylpolypyrrolidone. The resulting slurry was centrifuged at 12000*g* for 20 min. The supernatant was stirred with Amberlite XAD-4 and Dowex 1-X8 resins (0.2 g g^{-1} fresh weight each) and then filtered through 20-μm nylon cloth. HMKMT was purified from this crude homogenate through the chromatofocusing step as described previously (4). For time course experiments, at the indicated times after treatment with 5 mM $CuCl_2$, crude homogenates were prepared as described above from roots and shoots (2 g fresh weight) and used directly for enzyme assays.

The HMKMT enzyme assays were carried out as described previously (4). Briefly, the enzyme preparation (135 μl) was incubated with 0.2 mM (+)-HMK, 0.2 mM [^{14}C]AdoMet (25 mCi $mmol^{-1}$), 0.5 mM $MgCl_2$, 9 mM DTT, 1 mM EDTA, and 50 mM Tris–HCl, pH 7.9, in a total volume of 150 μl. Reactions were started with the addition of (+)-HMK (2 μl of 15 mM stock in DMSO) and were continued for 20 min at 30°C. The reaction was stopped with 350 μl 0.3 M sodium citrate, pH 4, and 4.5 ml of 0.55% PPO in toluene. The two-phase system was agitated in a vortex mixer and the content of nonpolar radioactivity was measured directly by scintillation counting. One unit of enzyme activity (1 kat) was the amount producing 1 mol of pisatin per second under these conditions.

For cellular fractionation studies, root and shoot tissues were weighed, chopped with a razor blade, and ground with a cold mortar and pestle in Buffer A (100 mM Hepes, pH 7.7, 0.3 M sorbitol, 2 mM EDTA, 5 mM $Na_2S_2O_5$) (10 ml g^{-1} fresh weight) with 5 mM phenylmethylsulfonyl fluoride, pepstatin (5 μg/ml), leupeptin (5 μg/ml), and 0.5 g sand and 0.05 g polyvinylpolypyrrolidone g^{-1} fresh weight. All subsequent steps were carried out at 4°C. The resulting slurry was filtered through four layers of cheesecloth and centrifuged in an HB4 (swinging bucket) rotor at 1000*g* for 5 min. The pellets from this and subsequent centrifugations were solubilized or resuspended in 90 mM Tris–HCl, pH 7.9, 10 mM dithiothreitol, and 1 mM EDTA in preparation for HMKMT enzyme assays. The 1000*g* supernatant was recentrifuged at 10,000*g* for 30 min and the 10,000*g* supernatant was recentrifuged at 95,000*g* for 35 min. The pellet from centrifugation at 95,000*g* was suspended in Buffer A by spreading it around the sides of the tube with a glass rod and then the suspension was centrifuged again at 95,000*g*. Solid $(NH_4)_2SO_4$ was added to the first 95,000*g* supernatant to 85% saturation, and the solution was stirred for 1 h at 4°C before centrifugation at 10,000*g* for 30 min.

SDS–polyacrylamide gel electrophoresis. Samples were concentrated and equilibrated in 2 mM potassium phosphate, pH 6.8, 10 mM dithiothreitol, and 1 mM EDTA by centrifugal ultrafiltration (Centricon 30) and then boiled in SDS buffer (15). SDS–PAGE was run according to Dreyfuss (15) with protein molecular weight markers that were prestained with Coomassie blue plus unstained ovalbumin (M_r 45,000). Gels were uniformly 12% unless indicated otherwise. For time course and cell fractionation experiments, gels were stained with silver nitrate under basic conditions (16).

Immunizations. The M_r 43,000 protein used to immunize a male Flemish Giant/Chinchilla rabbit (Laboratory Animal Services, Cornell University) was obtained by SDS–PAGE of enzyme purified through the chromatofocusing step. Immediately after electrophoresis, the gel was soaked in 4 M sodium acetate and viewed with diffuse light on a black background. The clear band migrating between the M_r 45,000 clear ovalbumin marker and the M_r 39,000 prestained marker (carbonic anhydrase) was cut out, soaked for 30 min in 50 mM phosphate-buffered saline (PBS), pH 7.2, and ground with a mortar and pestle. The gel slice was forced through an 18-gauge needle several times and then mixed with an equal volume of complete Freund's adjuvant for the first injection series and with incomplete adjuvant for booster inoculations. Subcutaneous injection at multiple sites was made on alternate days over a 5-day period for each series. The four injection series were at 0, 2, 4, and 22 weeks, beginning 1 week after withdrawing preimmune serum. Approximately 100 μg of protein was used per injection series for the first three series, and 200 μg for the final one. Seventeen days after the first day of the final injection series, the rabbit was exsanguinated, blood was allowed to clot, and the serum was stored at −80°C with 0.01% NaN_3. Titer as determined by ELISA was still increasing slightly 2 days prior to exsanguination.

Inhibition of enzyme activity. For experiments with IgG, whole serum was preabsorbed with SDS polyacrylamide gel for 1 h in 10 mM sodium phosphate, pH 8.2, and 0.15 M sodium chloride prior to loading onto a Protein A–Sepharose column equilibrated with the same buffer. The bound IgG was eluted with 0.1 M sodium citrate, pH 3.0, and the buffer was immediately equilibrated with 75 mM Tris–HCl, pH 8.0, and 1 mM EDTA (Buffer B).

The HMKMT enzyme, purified through the 48–80% ammonium sulfate precipitation (4), was equilibrated with Buffer B containing 10 mM dithiothreitol. A 15-μl reaction, containing 5 μl enzyme preparation, 10

μl whole serum, or up to 10 μl IgG and Buffer B to volume, was incubated for 1.5 h at 37°C. These immunoreactions were used directly for enzyme assays.

Western blots. Western blotting to nitrocellulose membranes was carried out according to the method of Towbin *et al.* (17). Gels were soaked in transfer buffer for 30 min prior to setting up the blotting apparatus. After transfer, blots were blocked in PBS and 2% nonfat dry milk. Primary antibody dilutions (1:500) were in PBS, 1% Tween 20, and 2% milk. The subsequent wash and incubation with goat anti-rabbit–alkaline phosphatase conjugate (1:1000) was in PBS, 0.05% Tween 20, and 2% milk. The final washes were in PBS and 0.05% Tween 20. Color development followed in 20 mM Tris-buffered saline, pH 7.5, with 0.5 mM $MgCl_2$.

The HMKMT antiserum was affinity-purified as follows: nitrocellulose was dipped in a solution of the 95,000*g* supernatant. The nitrocellulose was washed in three changes of PBS, 1% Tween 20, and 2% milk before incubation with whole HMKMT antiserum. After further washes, the bound antibodies were eluted with 1 vol 0.2 M glycine–HCl for 2 min. The eluant was immediately neutralized with 3.6 vol 0.12 M K_2HPO_4, pH 9.0 (18). This eluant constituted the affinity purified antibody, which was further diluted in PBS, 1% Tween 20, and 2% milk for use in Western blot analysis.

RNA preparations. Root and shoot tissue from $CuCl_2$-treated etiolated pea seedlings (4 g fresh weight) was ground in liquid N_2 with a mortar and pestle. The powder was scraped into H_2O-saturated phenol (2 ml g^{-1} fresh weight) containing 0.1% 8-hydroxyquinoline and mixed well before adding an equal volume of 0.1 M Tris–HCl, pH 9.0. The mixture was ground using a Polytron (Brinkman Instruments), and then the mixture was shaken vigorously at 50°C for 30 min. The supernatant obtained from centrifuging the mixture at 10,000*g* for 30 min in a swinging bucket rotor was transferred to a tube containing 12 ml phenol for the second phenol extraction. After two phenol extractions, the aqueous phase was extracted three times with an equal volume of $CHCl_3$ before the RNA was ethanol precipitated in the presence of 0.2 M sodium acetate. The pellet was washed three times by vortexing vigorously in 10 ml of 0.3 M sodium acetate, pH 5.5, and then dissolved in 0.3 M NaCl. DNA was pelleted by centrifugation at 5000*g* for 5 min. The supernatant was transferred to another tube and 2.5 vol absolute ethanol (−20°C) was added to precipitate the RNA. The yield of RNA was 1.3 to 3.9 mg per sample, with a 260/280 absorbance ratio of 1.6 to 1.8. RNA was precipitated twice more with ammonium acetate prior to being used to drive *in vitro* translation reactions.

In vitro translations, immunoprecipitations, and fluorography. *In vitro* translation reactions, containing 4 μl RNA (24 μg), 1 μl *trans*-^{35}S label (approximately 1 μCi), and 15 μl rabbit reticulocyte lysate, were incubated at 30°C for 70 min and stopped by the addition of 8 μl 10% SDS. After mixing the reactions well and heating them at 70°C for 3 min, Buffer C (472 μl) was added. Buffer C contained 20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.2% Triton X-100. For TCA precipitations, 8 μl was spotted in duplicate on Whatman 540 discs. These were air dried and then dropped into cold 5% TCA which was brought to a boil. After additional washing with cold 5% TCA and 95% ethanol, the radioactivity on the discs was determined by liquid scintillation counting in EcoScint. RNA-dependent radioactivity was the remainder after subtraction of the radioactivity bound to discs spotted with *in vitro* translation reactions containing no added RNA.

For immunoprecipitations, equal amounts of RNA-dependent TCA-precipitable counts were used in all samples of a set. The translation products (150 to 250 μl) were incubated with 1 to 20 μl antisera at room temperature on a gentle shaker for 1 h. The mixture was transferred to a tube containing 4 to 6 mg Protein A–Sepharose that had been swollen in Buffer C and absorbed with immunoglobulin-free bovine serum albumin. After gentle shaking for 1 h at room temperature, the gel was centrifuged and the resulting pellet was washed three times with Buffer C. Immunoprecipitated, radiolabeled products were solubilized by boiling with SDS buffer and then loaded onto gels for SDS–PAGE.

Gels were prepared for fluorography (19) by fixing in glacial acetic acid and then soaking in 26.2% 2,5-diphenyloxazole in glacial acetic acid. After washing in several changes of H_2O, gels were dried. Radioactivity in the gels was detected using Kodak X-OMAT film at −80°C.

RESULTS AND DISCUSSION

Antiserum raised against the HMKMT protein inhibited HMKMT enzyme activity (Table I). However, whole antiserum apparently contained factors other than antibodies which also inhibited HMKMT enzyme activity. Above this background, immune serum inhibited the enzyme 22% relative to the activity observed in the presence of preimmune serum from the same rabbit. The "nonspecific inhibitors" in whole serum were removed when IgG was purified by Protein A–Sepharose chromatography, and up to 63% specific inhibition of HMKMT activity by the purified IgG was observed. In contrast, purified IgG prepared from preimmune serum or prepared commercially did not inhibit the enzyme activity.

The HMKMT was highly soluble. The enzyme protein was detectable almost exclusively in the 95,000*g* supernatant of pea tissue fractionated by differential centrifugation (Fig. 1A). This fraction contained 92% of the enzyme activity, with the remaining activity distributed among the fractions as follows: 1000*g* pellet, 1%; 10,000*g* pellet, 5%; and 95,000*g* pellet, 2%. The immune serum had high specificity for the M_r 43,000 HMKMT in the 95,000*g* supernatant. However, this serum cross-reacted with proteins in the 10,000*g* pellet, several of which were recognized by the preimmune serum (Fig. 1B). Some of the cross-reactivity observed with immune serum might have developed as a result of physiological stress to the rabbit due to injection with SDS–polyacrylamide gel and the lengthy period over which immunization occurred.

TABLE I

Inhibition of HMKMT Enzyme Activity by Antibodies against the M_r 43,000 Protein

	HMKMT activity[a]				
	Whole serum		IgG		
Source of antisera	pkat[b] (±3%)	Relative activity	μg/rxn	pkat[b] (±3%)	Relative activity
None	2.2	100		2.9	100
Preimmune	1.4	64	47	3.2	110
			94	2.9	98
Immune	1.1	50	13	2.9	100
			52	2.2	75
			85	1.1	37
Sigma rabbit[c]			46	2.9	100
			92	2.9	99

[a] HMKMT was purified through the $(NH_4)_2SO_4$ precipitation step (4).

[b] One unit of enzyme activity (1 kat) was the amount producing 1 mol of pisatin per second under the conditions of the assay.

[c] Rabbit IgG purchased from Sigma Chemical Company.

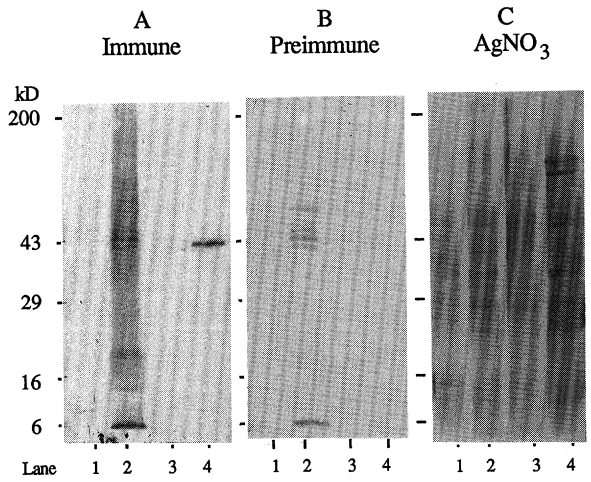

FIG. 1. Immunoblot analysis of the HMKMT protein in $CuCl_2$-treated pea tissue fractionated by differential centrifugation. (A) Western blot reacted with immune sera (HMKMT antisera). (B) Western blot reacted with preimmune sera from the rabbit used to raise the HMKMT antisera. (C) Silver nitrate staining of SDS–polyacrylamide gel. A total of 10 μg protein was loaded per lane for Western blots and 5 μg protein per lane for $AgNO_3$ staining. Lane 1, 1000*g* pellet; lane 2, 10,000*g* pellet; lane 3, 95,000*g* pellet; and lane 4, 95,000*g* supernatant.

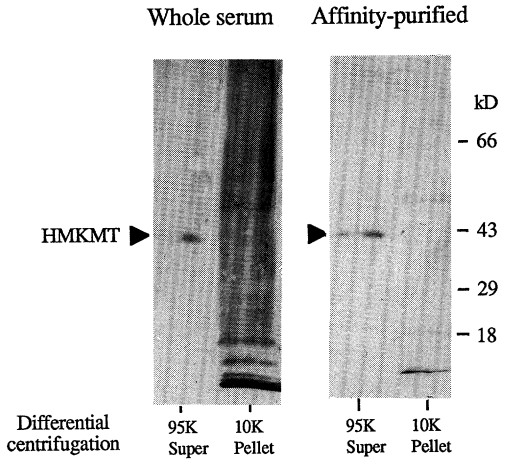

FIG. 2. Affinity purification of the HMKMT antisera removed nonspecific antibodies which reacted with proteins in the 10,000*g* pellet. SDS–PAGE on 4 to 20% gradient gels was carried out with 80 μg of protein per lane from the 10,000*g* pellet or 10 μg protein per lane from the 95,000*g* supernatant. Separate panels of the Western blot of this gel were incubated with whole HMKMT antiserum assayed at a 1:500 dilution or with affinity-purified serum assayed at 1:40.

Silver nitrate staining indicated that the lanes contained many proteins in addition to those recognized by the antisera (Fig. 1C).

The HMKMT antiserum was affinity-purified, since its intended uses are in gene cloning and immunocytolocalization, where its specificity is important. Affinity purification of the antisera reduced cross-reactivity almost entirely while the antisera still recognized the M_r 43,000 HMKMT in the 95,000*g* supernatant (Fig. 2). Possibly four bands remain cross-reactive—the major band is about M_r 14,000 in size and there are faint bands of higher molecular weight.

These results would seem to suggest that the subcellular location of pisatin biosynthesis is the soluble cytoplasm. However, phenylalanine ammonia-lyase showed similar distribution to HMKMT on Western blots of fractions prepared by differential centrifugation (not shown); PAL is considered to be associated with the endoplasmic reticulum, primarily on the basis of detectable channeling with cinnamate 4-hydroxylase, itself clearly an endoplasmic-reticulum-localized enzyme (20, 21). In addition, other workers reported the association of caffeoyl and feruloyl *O*-methyltransferases with the endoplasmic reticulum fraction on sucrose step gradients (22).

The HMKMT antisera cross-reacted with a M_r 66,000 protein present in HMKMT preparations purified through the gel filtration step (4, data not shown). This M_r 66,000 protein had emerged as a candidate for the HMKMT in highly purified preparations on the basis of cochromatography with enzyme activity. Although it did not photoaffinity label with [^{3}H]AdoMet (4), its immunological cross-reactivity suggests that this band represented inactive enzyme that was artificially polymerized. The fact that this M_r 66,000 band was not detected on blots of fresh (not highly processed) enzyme preparations, such as those used in the experiments presented here, supports this hypothesis.

The HMKMT enzyme protein was induced in pea roots by treatment with $CuCl_2$ (Fig. 3A). A low level of the protein was present in the "0" h sample as was a low level of HMKMT enzyme activity. In this experiment, HMKMT-specific enzyme activity increased from 0.33 to 6.2 nkat g^{-1} protein, peaking in the 16 h sample. The

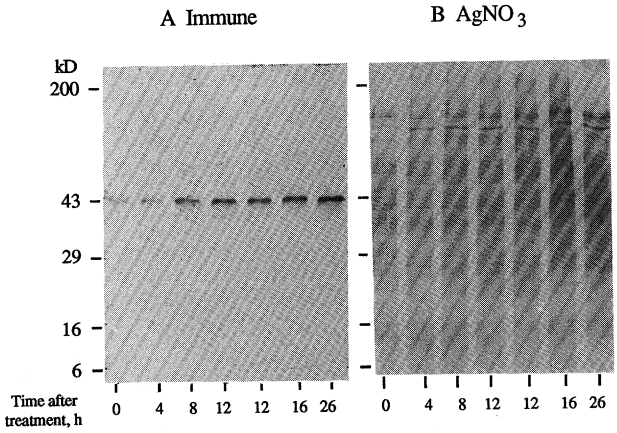

FIG. 3. Induction of the HMKMT protein by treatment of peas with $CuCl_2$. A total of 10 μg protein (10,000*g* supernatant) was loaded per lane for Western blot and 5 μg protein per lane for $AgNO_3$ staining. (A) HMKMT antisera. (B) Silver nitrate staining of SDS–polyacrylamide gel.

measurable level of enzyme activity in untreated tissue might be constitutive or might be stress-related due to (a) low grade infection of the tissue or (b) induction by physiological stress, similar to the induction of the bean CHS promoter in transgenic tobacco in the region of lateral root emergence (23). The lanes on the gel contained many proteins; an increase in abundance of the M_r 43,000 HMKMT protein in extracts of induced tissue was not evident by staining with $AgNO_3$ (Fig. 3B).

The HMKMT antisera specifically immunoprecipitated a M_r 43,000 protein from *in vitro* translation mixtures driven by total RNA from $CuCl_2$-treated peas (Fig. 4). No radioactivity was detected in lanes without antiserum nor in lanes of translation products immunoprecipitated with preimmune serum. The PAL antisera (12) precipitated a triplet of proteins in the M_r 68,000 to 77,000 range, as expected for PAL. In some translation reactions, a broad, faint band around M_r 50,000 was also immunoprecipitated by PAL antisera (Fig. 4). Partial degradation of PAL during *in vitro* translation reactions has been reported (24) and the protein of M_r 50,000 may be the product of the degradation of PAL as well.

$CuCl_2$ treatment induced an increase in the HMKMT mRNA translational activity which was detectable 2 h after treatment and continued to rise for 8 to 10 h (Fig. 5). Peak mRNA translational activity preceded peak enzyme activity by about 6 h. mRNA translational activity

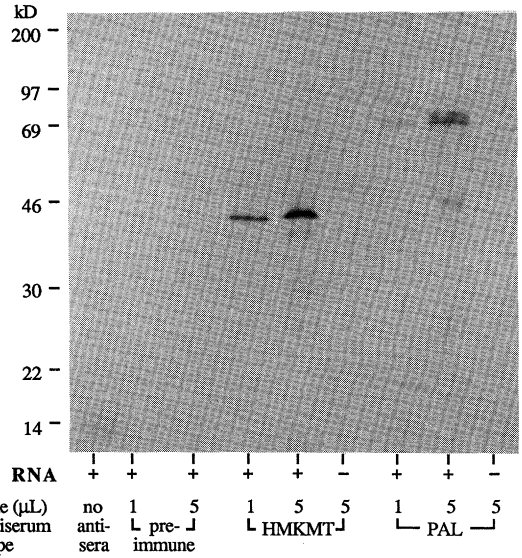

FIG. 4. *In vitro* translation and immunoprecipitation of the M_r 43,000 protein by the HMKMT antiserum and of a M_r 68,000 to 70,000 triplet by PAL antisera. Total RNA extracted 12 h after treatment of etiolated peas with $CuCl_2$ was translated with a rabbit reticulocyte lysate system as described under Materials and Methods. Translation products (200-μl aliquots) were immunoprecipitated with indicated amounts of HMKMT antisera, preimmune sera from the same rabbit, or PAL antisera (12), followed by Protein A–Sepharose (bovine-serum-albumin-treated), and subjected to SDS–PAGE and fluorography as described under Materials and Methods.

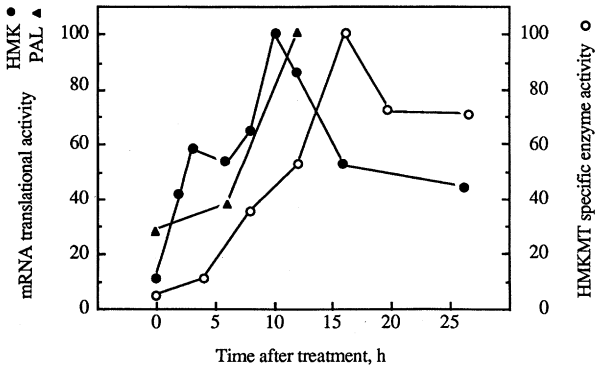

FIG. 5. Induction of HMKMT and PAL mRNA translational activities and HMKMT enzyme activity by $CuCl_2$ treatment of peas. *In vitro* translation and immunoprecipitation of total RNA was followed by SDS–PAGE and fluorography as described in Fig. 4. Autoradiographs were used as templates to locate radiolabeled bands on gels; bands were excised and their radioactive contents determined by liquid scintillation counting. mRNA translational activities and HMKMT specific enzyme activities (nkat g^{-1} protein) are each expressed relative to the maximum value observed.

for PAL, the first enzyme of the phenylpropanoid pathway, was induced concurrently. The time course of induction of PAL active mRNA by treatment with $CuCl_2$ was similar to that observed when eliciting pisatin biosynthesis in peas by treatment with actinomycin D (25).

It is known that PAL mRNA translational activity induced by stress in bean (*Phaseolus vulgaris* L.) and parsley (*Petroselinum hortense* L.) reflects activated gene transcription (26, 27). A similar correlation between the stress-induced increase in active mRNA abundance and gene expression has been found for 4-coumarate CoA ligase and chalcone synthase. Thus, it is reasonable to suggest that the induction of HMKMT mRNA by stress in garden pea is indicative of increased transcription of the HMKMT gene(s), and it would appear likely that gene expression along the entire phytoalexin biosynthetic pathway, from the first committed step, catalyzed by PAL, to the terminal step, catalyzed by HMKMT, is coordinately induced in garden peas by $CuCl_2$ treatment. This coordinate induction, suggested for the earliest enzymes of the pathway (28), would then be extended along an entire branch of a complex biosynthetic pathway.

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